Role of circumsporozoite protein-specific T-cells in protective immunity against *Plasmodium berghei*

U. Krzych,[1,2] K. White,[1,2] H.T. Link,[1,2] J. Sadoff,[3] D. Lanar,[2] W.R. Ballou,[4] T. Jareed,[2] & A. Agarwal[3]

The Plasmodium berghei *circumsporozoite (CS) protein-specific T-cell repertoire was analysed in C57Bl/6 (H-2^b), Balb/c (H-2^d), and C3H/HeN (H-2^k) mice immunized with irradiated sporozoites and the proliferative responses were correlated with the protective status of each strain. Splenic lymphocytes responded to the priming antigen, but the responses varied according to both murine strain and immunization schedule. Analysis of cytolytic T lymphocyte (CTL) responses in mice immunized with irradiated* P. berghei *sporozoites or with a* Salmonella*-recombinant CS protein construct revealed that each immunization induces CTLs recognizing different epitopes on the target cells. The variations in immune reactivities among different murine strains to the CS protein antigens and the variations in the responses to the authentic versus recombinantly expressed CS protein suggest that distinct immune mechanisms may be involved in rendering immune protection. Furthermore, the molecular context of the immunizing antigen may influence the outcome of the fine specificity of T cells involved in immune protection.*

Introduction

Complete immune protection against infection with *Plasmodium berghei* sporozoites can be induced by immunization with irradiated sporozoites of the homologous strain (*1*, *2*), and partially by oral inoculation of the *Salmonella–P. berghei* recombinant CS protein construct (*3*, *4*). Studies have shown that the circumsporozoite (CS) protein contains the immunodominant B-cell determinant(s) localized within the central repeat region of the CS protein (*5*, *6*). Immune protection is mediated in part by anti-CS antibodies, since anti-CS monoclonal antibodies can transfer protection (*7*, *8*). Cell-mediated immunity has also been shown to confer protection as demonstrated *in vivo* by depletion of selected T-cell subpopulations (*9*, *10*). Despite these observations, the protective immune mechanism is incompletely understood, particularly concerning the role of CS protein-specific T-cells arising from immunization with irradiated sporozoites. Investigations of cellular immune processes occurring during the protective phase will permit a better understanding of anti-sporozoite immunity necessary for vaccine development. In the present study we examined different mouse strains capable of generating protective immunity against live sporozoite challenge and analysed the CS protein-specific T-cell proliferative and cytolytic repertoires induced by i.v. immunization with irradiated sporozoite antigens or by oral administration of live attenuated *Salmonella*-recombinant vaccine expressing *P. berghei* circumsporozoite antigens.

[1] Department of Biology, Catholic University of America, Washington, DC 20064, USA.

[2] Department of Immunology, Walter Reed Army Institute of Research, Washington, DC 20307-5100, USA. Correspondence should be sent to Dr U. Krzych at this address.

[3] Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, DC, USA.

[4] Current address: Univax, Rockville, MD, USA.

Materials and methods

Mice. Female Balb/c (H-2^d), C57Bl/6 (H-2^b) and male and female C3H/HeN (H-2^k) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and used at 6–12 weeks of age.

Sporozoite preparation. Sporozoites dissected from the salivary glands of infected *Anopheles stephensi* mosquitos were provided by Dr Imogene Schneider, Dept. of Entomology, Walter Reed Army Institute of Research (WRAIR). Upon dissection, sporozoites were placed in Medium 199 (Gibco) containing 3% normal mouse serum (NMS). For immunizations, aliquots of sporozoites were irradiated at 15 000 rad from a cobalt source and stored on ice until the time of immunization. Sporozoite antigen used to stimulate lymphocyte cultures was irradiated at 30 000 rad and stored at -70 °C until use.

Mosquito salivary gland antigen. Non-infected mosquito salivary glands were sham dissected and the debris treated in an identical manner to sporozoites. Gland debris was used at a concentration equivalent to 10^4 sporozoites/gland.

Construction of* Salmonella–P. berghei *CS recombinants. The transformation of full length CS gene of *P. berghei* strain NK65 into *Salmonella typhimurium* WR4024 trp R– was described in detail previously (*4*).

Immunizations. All mouse strains were immunized intravenously (i.v.) with 75 000 irradiated sporozoites followed by two i.v. boosts of 20 000 irradiated sporozoites one week apart. Representative mice were challenged i.v. with 10 000 live sporozoites. For some experiments, separate groups of Balb/c mice were immunized orally with *Salmonella–P. berghei* CS recombinant constructs as described previously (*4*).

Parasitaemia screening and protection. Individual thin blood smears were taken daily from day 5 through day 14 post challenge and stained with Giemsa. One hundred oil-immersion fields were viewed for parasitized erythrocytes. Mice were considered protected if parasites were not detected after 14 days.

Complete culture media. Cell cultures were maintained on complete media consisting of RPMI 1640 mixed with EHAA Medium (1:1 v/v) (Microbiological Associates) and enriched with 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), 4 μmol glutamine (Gibco), 2.5 μmol sodium pyruvate (Gibco), 10 μmol non-essential amino acids (Gibco), 50 mmol 2-mercaptoethanol (Biorad), and 0.5% NMS (normal mouse serum) or 5% fetal bovine serum (FBS).

Proliferative assay. Unfractionated splenic cell populations were cultured in 96-well microtitration plates (Costar) at a final concentration of 5×10^5 cells/0.2 ml in complete medium containing sporozoite antigen ranging from 300 to 3000 frozen/thawed/30 000 rad-irradiated sporozoites, or equivalent amount of mosquito gland debris, or *Salmonella–P. berghei* CS protein constructs. Control cultures contained medium alone. Cells were cultured for 5 days at 37 °C in a humidified atmosphere containing 5% CO_2, and during the last 16 hours of culture 1 μCi of tritiated thymidine (^{3}H-TdR, NEN, DuPont) was added to each well to quantitate proliferative activity. Cultures were harvested using an automated sample harvester (LKB Betaplate) and analysed by scintillation spectroscopy (LKB Betaplate).

Cytolytic T lymphocyte (CTL) assay. Sporozoite-immune spleen cells from Balb/c and C57Bl/6 mice and *S. typhimurium* (WR4204)–*P. berghei* construct-immune Balb/c mice were analysed for CTL response directed against the CS protein expressed on NIH-3T3 cells transfected with the *P. berghei* CS gene or on P815 cells infected with a pseudorabies viral construct containing the *P. berghei* CS gene (P815-PRV-CSP) (gift from Dr Christina Cheng, Syntro, San Diego, CA). Graded numbers of spleen cells were mixed with the ^{51}Cr (100 μCi/10^7 cells) labelled target cells at effector to target (E/T) ratios of 3:1 to 30:1 in 96-well plates in 0.2 ml complete medium and incubated for 4 h at 37 °C in a 5% CO_2 humidified atmosphere. At the end of the incubation period the supernatants were harvested and assayed for ^{51}Cr activity. Naïve spleen cells and *Salmonella*-immune spleen cells were used as effector controls while NIH-3T3 and P815 cells were used as control target cells. The values represent the mean of triplicate wells and the percentage specific lysis was calculated by the following:

$$\frac{[\text{experimental release (cpm)} - \text{spontaneous release (cpm)}]}{[\text{total (cpm)} - \text{spontaneous release (cpm)}]} \times 100$$

Transfection of NIH-3T3 cells. A 1.7 Kb fragment containing the entire 1.4 Kb coding region of the *P. berghei* CS gene and a 0.3 Kb 5′ noncoding sequence was cloned into the Hind III site of pCDM8 expression vector containing a CMV/T7 promoter region, SV40 origin, splice region, and polyadenylation signal downstream (*11*). P3-competent bacteria were transformed with the pCDM8-CS vector and screened by colony hybridization to the ^{32}P-TdR labelled 1.4 Kb CS probe. NIH-3T3-fibroblast cells were co-transfected with 10 μg purified pCDM8-CS and 1 μg purified pSV2Neo by calcium phosphate precipitation. Briefly, 5×10^5 NIH-3T3 cells (per 60-mm petri-dish) were incubated with the calcium phosphate/DNA precipitate for 6 h after which the DNA precipitate was removed and the cells treated with 10% glycerol for 1 min followed by extensive washing with PBS. Cells were incubated in complete medium for 2 days. Transfected cells were selected by culturing in 2 mg/ml G418 (geneticin, Gibco) for 10 days and stable transfectants were maintained in 0.2 mg/ml G418. Transfected cells expressing the CS gene were screened by total transfected cellular RNA northern blot analysis using the ^{32}P-TdR labelled 1.4 Kb CS probe. Transfected clones were obtained by limiting dilution of northern blot-positive cell lines, and were screened for the expression of CS mRNA by northern blot analysis.

Pseudorabies virus–P. berghei CS construct infection of P815 cell. P815 cells were grown in complete medium as described and supplemented with 10% FBS. Twenty-four hours before the CTL assay, 10×10^6 P815 cells/ml were infected with 10^7 viral plaque-forming units (PFU). The extent of infection was verified microscopically and expression of the CS protein was ascertained by western blot analysis of homogenized cell preparations.

CD4⁺ and CD8⁺ T-cell depletions. Splenic cell populations (20×10^6 cells/ml) were depleted of $CD4^+$ T cells or $CD8^+$ T cells using a 1:3 dilution of culture supernatants of the hybridoma 2RL (MAb 2RL) specific for CD4, or hybridoma 83-12-5 (MAb 83-12-5) specific for CD8 (both gifted by Dr Richard Hodes, NIH) for 1 hour at 4°C. Cells were pelleted and resuspended in Low Tox Rabbit Complement (Cedar Lane Laboratories, Westbury, N.Y.) at a 1:10 final dilution in HBSS containing 1% FBS, and incubated at 37°C for 1 hour. Cells were centrifuged at $400 \times g$ for 10 minutes and washed three times in HBSS. $CD4^+$ and $CD8^+$ enriched populations were resuspended at 5×10^6 viable cells/ml in complete medium.

Results

Sporozoite-induced spleen cells proliferate to sporozoite antigens in culture. Spleen cells from Balb/c ($H\text{-}2^d$), C57Bl/6 ($H\text{-}2^b$) and C3H/HeN ($H\text{-}2^k$) mice, previously immunized i.v. with 75 000 irradiated sporozoites followed by two i.v. boosts of 20 000 irradiated sporozoites one week apart, were cultured with irradiated, frozen/thawed sporozoite antigens. This particular immunization regimen was chosen for analysis since it usually induces protection against a challenge with 10 000 live sporozoites (*1*, *2*). Balb/c mice always showed a stronger anti-sporozoite proliferative response than C57Bl/6 strain, whereas C3H/HeN did not respond to sporozoite antigen (Table 1). The variability in the proliferative responses suggested a strain-dependent sensitivity to sporozoite antigens. As shown in the Table, the proliferative response was sporozoite-specific, as graded amounts of mosquito salivary gland debris, obtained from a sham sporozoite dissection of uninfected mosquitos, induced only baseline levels of proliferative activity. In an earlier study, lymph node cells from Balb/c and C57Bl/6 mice primed with complete Freund's adjuvant (CFA) did not respond to sporozoite antigen *in vitro*, demonstrating that the irradiated sporozoites were not mitogenic (*12*). Moreover, spleen cells from naïve animals did not respond to sporozoite antigens in culture (data not shown).

Dissociation of protection and proliferative T-cell activity. To investigate a possible relationship between the proliferative response and protection, Balb/c, C57Bl/6, and C3H/HeN mice were immunized with either a single dose of 75 000 irradiated sporozoites or with one or two boosting doses of 20 000 irradiated sporozoite following priming. Pooled splenic lymphocytes from each group (3–5 mice/group) were assayed *in vitro* for sporozoite-specific proliferative responses in the presence of graded amounts of sporozoite antigen. Additionally, groups of 5 mice from each strain, were challenged i.v. with 10 000 live sporozoites and subsequently screened for parasitaemia.

Sporozoite-specific responses were consistently observed in cultures of splenic lymphocytes from Balb/c and C57Bl/6 strains, although the magnitude of reactivities differed in each strain. A low level of proliferative reactivity to sporozoite antigens was seen in the Balb/c spleens after the priming dose that induced full protection in these mice. In contrast, C57Bl/6 mice mounted a high proliferative activity after a priming dose of 75 000 irradiated sporozoites. However, they were not protected until a third dose of 20 000 sporozoites, at which time the proliferative activity was diminished to a 3-fold increase over the background responses. Generally, proliferation of sporozoite-immune C3H/HeN splenic lymphocytes was not observed, but protection was induced in most mice in 3 out of 4 experiments using the full 3-dose immunization schedule (Table 2). Interestingly, splenic populations obtained from mice immunized with the *Salmonella–P. berghei* CS construct failed to proliferate to all malarial antigens tested, including sporozoites, CS protein synthetic peptides, and recombinantly expressed CS protein (data not shown).

Clearly, these data show variations in the sensitivities of different mouse strains to the immunizing doses of sporozoites eliciting immune protection and proliferative activity. Furthermore, the observed reverse correlation between immune protection and proliferative responses is not well understood, but it is possible that functionally unique T-cell types may be generated in each strain following a particular immunization regimen, causing the observed fluctuations.

Table 1: **Sporozoite (SPZ) antigens induce specific responses in sporozoite-immune spleen cells**

Mouse strain	No. of sporozoites for immunization ($\times 10^3$)	*In vitro* splenic proliferative responses (delta cpm $\times 10^3$)	
		SPZ antigens	Mosquito debris
Balb/c	75, 20, 20	17.8	1.9
C57BL/6	75, 20, 20	9.0	0.7
C3H/HeN	75, 20, 20	1.0	0.5

Sporozoite and* Salmonella–P. berghei *immunizations induce CS protein specific CTLs. As shown in Table 3, Balb/c mice immunized with irradiated sporozoites generated CTLs specific for 3T3 transfected CS protein targets. CTL activity was detected in both the unfractionated splenic population and to a lesser degree (10%) in the $CD8^+$ enriched population, but not in the $CD4^+$ enriched population; 15% lysis was detected in the $CD8^+$ enriched population using P815-PRV-CSP targets. Interestingly, immunization with *Salmonella–P. berghei* CS construct only primed for CTLs able to recognize and lyse P815-PRV-CSP and not 3T3-CS protein targets. Once again, the CTL activity was apparent only in the whole spleen and $CD8^+$ enriched populations. In parallel experiments with C57Bl/6 mice immunized with irradiated sporozoites CTL activity was not detectable (data not shown).

Discussion

Analysis of the *P. berghei* sporozoite-specific T-cell repertoire was initiated by investigating whether immunization with sporozoites induces T cells responsive to sporozoite antigen in culture. An earlier report (*13*) suggested that *P. berghei* sporozoites are mitogenic for murine T cells. In contrast, in this and other reported studies (*12*) we found that sporozoite-immunized splenic lymphocytes responded *in vitro* specifically and in a dose-dependent fashion to antigens from sporozoites treated by freeze/thaw and irradiation with 30 000 rad.

Upon boosting immunizations with one or two doses of 20 000 irradiated sporozoite, Balb/c mice became high proliferative responders, maintaining their protective status, while proliferative responses of C57Bl/6 mice diminished to lower levels although they developed protective immunity. It is not entirely clear why the onset of protection is accompanied by depressed lymphoblastic responses, although a decrease in the IL-2R density on lymphocytes was partly responsible for similar results in other systems (*14*, *15*).

In contrast to results obtained with Balb/c and C57Bl/6 mice, the nonresponsiveness of C3H/HeN spleen cells to sporozoite antigen was persistent and reproducible in all experiments. The nonreactivity to sporozoite antigens was also observed in A/J mice,

Table 2: **Strain-dependent requirements for induction of anti-sporozoite (SPZ) protection and antigen-specific activation of splenic lymphocytes**

Strain	No. of sporozoites for immunization ($\times 10^3$)	% protected	*In vitro* anti-SPZ proliferative responses (delta CPM $\times 10^3$)
Balb/c	75	100	3 ± 0.5
	75, 20	100	17 ± 2.1
	75, 20, 20	100	16 ± 2.5
C57Bl/6	75	0	29 ± 4.0
	75, 20	40	16 ± 2.2
	75, 20, 20	100	9 ± 3.5
C3H/HeN	75	0	0.8 ± 0.3
	75, 20	60	0.8 ± 0.2
	75, 20, 20	100	1.1 ± 0.3

Table 3: **Cytolytic activity of sporozoite immune splenic populations**

In vivo priming	Effector[a] cells	% specific lysis of: P815-PRV-CSP	P815	3T3-CS	3T3
SPZ	WSPLC	5	1	25	5
SPZ	$CD4^+$	1	1	5	5
SPZ	$CD8^+$	15	1	10	1
WR2024	WSPCL	60	1	10	10
WR2024	$CD4^+$	5	1	10	10
WR2024	$CD8^+$	20	1	1	1
C-V	WSPCL	0	0	0	0
NAIVE	WSPCL	0	0	0	0

[a] E/T = 30:1.

and both strains are not identical at all, except for one locus (D) of the major histocompatibility complex. Although some important information is already available about the T-cell involvement in anti-sporozoite protective immunity, we still do not have a full understanding about the fine cellular mechanisms involved in immune protection to sporozoites. The observations reported here strongly suggest the involvement of multifactorial mechanisms, whereby not only unique T-cell specificities, but also diverse T-cell functions may be induced. For example, $CD8^+$ T cells may suppress proliferative $CD4^+$ T cells in the C3H/HeN and A/J strains, yet they may function as cytolytic T cells in Balb/c mice. The induction of presumably diverse functional T cells in different mouse strains may be triggered by minor antigenic variations among *P. berghei* sporozoites from the uncloned NK65 *P. berghei* strain that we used. An important degree of variability in the gene coding for the CS protein has been noted within different strains of many *Plasmodia* species (*16*, *17*).

The molecular context of the CS protein antigen and mouse strain also appeared to affect detectable CTL activity. Whereas the authentic sporozoite-associated CS protein primed for CTLs predominately capable of recognizing CS protein expressed on PbCS gene transfected 3T3 targets, immunization with *Salmonella* CS construct induced cells directed against P815-*P. berghei* CS protein targets. Protein and total RNA analysis revealed transcription of the CS gene by the target cells, but the portion of the gene product being expressed is not known. The CTL activity was directed against some CS epitope as evidenced by the inability of either primed populations to lyse normal 3T3 or P815 targets. Also naïve or *Salmonella* control-vector primed mice were unable to lyse any of the targets. The CTL activity was detected immediately after dissection of spleens, without any further *in vitro* stimulation. This supports the idea that these CTLs are not artifacts of culturing techniques but rather are actually present *in vivo*. It is of interest that in multiple experiments CTL activity was only detected in Balb/c mice. It is conceivable that sporozoite priming of other mouse strains may not induce specific CTLs or that they may require additional *in vitro* stimulation with the appropriate antigen.

Supported by the apparent differences in the sporozoite-specific T-cell sets, these findings underscore the differences in *P. berghei* sporozoite-specific immune responses generated in different murine strains. Furthermore, these data demonstrate that identical antigens, like CS protein, presented in an authentic form or as a recombinant protein delivered by either bacteria or virus may induce T cells with diverse specificities and functions. We hypothesize that these functionally diverse T-cell specificities arise owing to different processing and presentation pathways used by each form of the antigen.

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